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Post-mortem culture of *Balamuthia mandrillaris* from the brain and cerebrospinal fluid of a case of granulomatous amoebic meningoencephalitis, using human brain microvascular endothelial cells

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The first isolation in the UK of *Balamuthia mandrillaris* amoebae from a fatal case of granulomatous amoebic meningoencephalitis is reported. Using primary cultures of human brain microvascular endothelial cells (HBMECs), amoebae were isolated from the brain and cerebrospinal fluid (CSF). The cultures showed a cytopathic effect at 20–28 days, but morphologically identifiable *B. mandrillaris* amoebae were seen in cleared plaques in subcultures at 45 days. The identification of the organism was later confirmed using PCR on Chelex-treated extracts. Serum taken while the patient was still alive reacted strongly with slide antigen prepared from cultures of the post-mortem isolate, and also with those from a baboon *B. mandrillaris* strain at 1 : 10 000 in indirect immunofluorescence, but with *Acanthamoeba castellanii* (Neff) at 1 : 160, supporting *B. mandrillaris* to be the causative agent. If the presence of amoebae in the post-mortem CSF reflects the condition in life, PCR studies on CSF and on biopsies of cutaneous lesions may also be a valuable tool. The role of HBMECs in understanding the interactions of *B. mandrillaris* with the blood–brain barrier is discussed.

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INTRODUCTION

Free-living amoebae such as *Naegleria*, *Acanthamoeba* and *Balamuthia* are potential pathogens with increasing importance in human health, and are known to produce two fatal diseases, primary amoebic meningoencephalitis (PAM) and granulomatous amoebic encephalitis (GAE). PAM, caused by *Naegleria fowleri*, is a fast progressing disease (lasting 7–14 days), affecting both immunocompromised and immunocompetent hosts. *N. fowleri* gains entry into the central nervous system (CNS) through the olfactory neuroepithelium, producing necrotizing and haemorrhagic meningoencephalitis, and is associated with stiff neck, headache, vomiting, fever and nausea (Barnett *et al.*, 1996; Ferrante, 1991; Ma *et al.*, 1990; Martinez & Visvesvara, 1997, 2001). In contrast, GAE can be caused by both *Balamuthia mandrillaris*

and *Acanthamoeba* species and is thought to be initiated by the entry of amoebae through the lower respiratory tract or skin lesions, followed by haematogenous spread (reviewed by Khan, 2003; Marciano-Cabral & Cabral, 2003). Circulating amoebae most likely gain access through the blood–brain barrier to enter the CNS (Khan, 2003). GAE is characterized by subacute or chronic granulomatous encephalitis with clinical features such as stiff neck, headache, fever, confusion, nausea and seizures (Marciano-Cabral & Cabral, 2003). These conditions can persist from several weeks to months with fatal consequences. The predisposing factors for GAE due to *Acanthamoeba* include alcoholism, drug abuse, steroid treatment, cancer chemotherapy, radiotherapy and organ transplantation (Anderlini *et al.*, 1994; Martinez & Janitschke, 1985; Sell *et al.*, 1997). In general, conditions that lead to immune suppression contribute to enhanced susceptibility to GAE due to *Acanthamoeba*. However, more worryingly, *B. mandrillaris* apparently produces GAE irrespective of the status of the host immune system. This is of particular concern as there are no definitive treatments available for

Abbreviations: CSF, cerebrospinal fluid; GAE, granulomatous amoebic meningoencephalitis; HBMECs, human brain microvascular endothelial cells; LDH, lactate dehydrogenase.

these fatal diseases. Recently, we have developed an *in vitro* model of the blood–brain barrier by isolating human brain microvascular endothelial cells (HBMECs) (Alsam *et al.*, 2003). Using this model, we have isolated *B. mandrillaris* from the brain necropsy as well as the post-mortem cerebrospinal fluid (CSF) of an immunocompetent GAE patient. These results were further confirmed using PCR and indirect immunofluorescence assays.

METHODS

Chemicals. All chemicals were purchased from Sigma, unless otherwise stated.

HBMECs. Primary BMECs of human origin were isolated and cultured as previously described (Alsam *et al.*, 2003). Briefly, HBMECs were purified by fluorescence-activated cell sorting, and their purity was tested using endothelial markers such as expression of F-VIII and carbonic anhydrase IV and uptake of acetylated low-density lipoprotein (AcLDL). The resulting cultures were more than 99 % pure. HBMECs were routinely grown on rat tail collagen coated dishes in RPMI (Invitrogen) containing 10 % heat-inactivated fetal bovine serum, 10 % Nu-Serum, 2 mM glutamine, 1 mM pyruvate, penicillin (100 U ml⁻¹), streptomycin (100 µg ml⁻¹), non-essential amino acids and vitamins.

***B. mandrillaris* cultures.** A standard isolate (ATCC 50209) of *B. mandrillaris*, isolated from the brain of a mandrill baboon, was routinely cultured on HBMEC monolayers. Briefly, 10⁶ amoebic trophozoites were inoculated on HBMEC monolayers in RPMI containing 2 mM glutamine, 1 mM pyruvate and non-essential amino acids. Flasks were incubated at 37 °C in a 5 % CO₂ incubator and observed daily. A complete HBMEC monolayer degradation was observed within 3–4 days when the amoebic numbers had reached approximately 5 × 10⁶, more than 99 % of which were trophozoites. These were subsequently used in PCR, immunofluorescence and cytotoxicity assays.

Isolation of *B. mandrillaris* from the post-mortem sample of the infected patient. The post-mortem samples, i.e. the brain necropsy and the CSF, were derived from a 33-year-old immunocompetent male who died of GAE subsequent to suffering from skin lesions which developed following a traffic accident in Bolivia. The patient had no history of HIV infection or of being immunocompromised and his CD4 count was only slightly depressed (621 cells µl⁻¹; normal range 775–1385 cells µl⁻¹). Both the brain necropsy and the CSF specimens were inoculated on HBMEC monolayers in tissue culture flasks. Flasks were incubated at 37 °C in a 5 % CO₂ incubator and observed daily for the presence of *B. mandrillaris* as previously described (Schuster, 2002).

DNA extraction. For PCR reactions, DNA extractions were performed as previously described (Khan *et al.*, 2001). Briefly, 3 × 10⁶ amoebae were harvested by centrifugation at 700 g for 10 min. The cell pellet was washed three times with PBS and finally resuspended in 0.5 ml PBS in microcentrifuge tubes. To this, 0.5 ml lysis buffer (100 mM KCl, 40 mM Tris, 5 mM MgCl₂, 1 % Tween and 200 µg proteinase K ml⁻¹) was added and tubes were incubated at 56 °C for 60 min followed by 10 min incubation at 100 °C. Finally the reaction mixtures were centrifuged at 2000 g for 5 min, and supernatant DNA was quantified and used as the PCR template.

For clinical purposes it will be important to have a sensitive assay which can identify *B. mandrillaris* at low cell numbers. Previously, we have shown that Chelex can be potentially used to isolate DNA from fewer cells as it does not involve washing steps or DNA precipitation which could result in the loss of the DNA (Khan *et al.*, 2001). Briefly, cell counts

were performed on the washed pellet (counted using a haemocytometer), volumes containing approximately 50, 100 and 1000 cells were placed in 0.5 ml microcentrifuge tubes and 30 µl Chelex (insta-gene matrix; Bio-Rad) was added to each tube. Tubes were incubated at 56 °C for 20 min and at 100 °C for 10 min and finally centrifuged at 2000 g for 5 min. The supernatant was used as the DNA template.

PCR analysis. *B. mandrillaris* genus-specific primers were used for the PCR amplification of the mitochondrial 16S rRNA gene as previously described (Booton *et al.*, 2003). Primer sequences were: 5' Balspec 16S, 5'-CGCATGTATGAAGAAGACCA-3'; and 3' Balspec 16S, 5'-TTACCTATATAATTGTCGATACCA-3'. Briefly, PCR was performed in a volume of 50 µl containing 1.25 U *Taq* polymerase (Qiagen), 0.2–0.4 µg DNA, 200 µM dNTPs, 2 mM MgCl₂ and 2 µM primer. The PCR reactions were performed at 94 °C for 1 min, 48 °C for 2 min and 72 °C for 3 min for 40 cycles with a final elongation step of 15 min at 72 °C. Amplified DNA was electrophoresed on a 1 % agarose gel, stained and visualized under UV illumination.

Indirect immunofluorescence assays for anti-amoebic antibody.

To detect anti-*B. mandrillaris* antibodies in the sera of the infected patient, indirect immunofluorescence assays were performed. Briefly, *B. mandrillaris* (baboon isolate) was collected by centrifugation at 2000 g for 5 min and resuspended in 10 ml PBS. This process was repeated three times. Finally, 100–500 *B. mandrillaris* trophozoites were dispensed in 5 µl PBS on Hendley Essex Multispot microscope slides (24 wells), allowed to dry, fixed with ice-cold 100 % acetone for 1 h, dried, and stored wrapped in tissue paper in a sealed box with silica gel at –20 °C until needed. On recovery from the freezer the wrapped slides were allowed to warm to room temperature in a desiccator. Wells were incubated in a moist box with dilutions of the patient's serum in PBS containing 2 % dried milk ('Marvel') for 30 min at room temperature, washed in PBS, incubated with 1/120 FITC-labelled goat anti-human polyclonal antibody (30 min, room temperature), and visualized under blue-light epifluorescence (Schuster *et al.*, 2003).

Cytotoxicity assay. To further determine the pathogenic potential of *B. mandrillaris* isolates used in this study as well as to develop an *in vitro* model to study *Balamuthia* pathogenesis, cytotoxicity assays were performed (Alsam *et al.*, 2003). Briefly, HBMECs were grown to confluency in 24-well plates. *B. mandrillaris* isolates (5 × 10⁵ amoebae per well) were incubated with cell monolayers in serum-free medium (RPMI 1640 containing 2 mM glutamine, 1 mM pyruvate and non-essential amino acids) at 37 °C in a 5 % CO₂ incubator for up to 48 h. At the end of this incubation period, supernatants were collected and cytotoxicity was determined by measuring lactate dehydrogenase (LDH) release (cytotoxicity detection kit; Roche Applied Science). Briefly, conditioned media of co-cultures of *B. mandrillaris* and HBMECs were collected and the percentage LDH was detected as follows: (sample value–control value/total LDH release–control value) × 100 = % cytotoxicity. Control values were obtained from HBMECs incubated in RPMI alone. Total LDH release was determined from HBMECs treated with 1 % Triton X-100 for 30 min at 37 °C.

RESULTS

Amoebae isolated from the GAE patient were identified as *B. mandrillaris* based on morphology

The post-mortem brain and CSF specimens from the GAE patient were inoculated on HBMEC monolayers and observed daily for the presence of *B. mandrillaris*. Within 20–28 days of incubation, HBMEC monolayers were disrupted; however, no amoebae were observed. Supernatants were collected and inoculated on fresh HBMEC monolayers. On

day 40–45, the presence of a large number of *B. mandrillaris* trophozoites (based on morphological characteristics, in particular movement by characteristic pseudopods) was observed (Fig. 1). Upon further incubation, we observed transformations of the trophozoites into cysts (Fig. 1). Cysts were further inoculated on HBMEC monolayers to obtain large numbers of *B. mandrillaris* to use in all subsequent assays.

PCR confirms clinical isolate as *B. mandrillaris*

To confirm the identification of amoebae isolated from the post-mortem specimens as *B. mandrillaris*, PCR methods were employed using *Balamuthia*-specific primers (Booton *et al.*, 2003). These primers are derived from the mitochondrial 16S rRNA gene (*rns*) and specifically amplify a product from the whole-cell DNA of *B. mandrillaris* but not from other amoebae such as *Acanthamoeba*, indicating their potential value as a diagnostic tool (Booton *et al.*, 2003). Using this primer pair, we yielded a single expected PCR product of 1075 bp, further confirming the clinical isolate as *B. mandrillaris* (Fig. 2a, lanes 3 and 4). The DNA isolated from *B. mandrillaris* (baboon) was used as a positive control

and gave a similar PCR product (Fig. 2, lane 2). Moreover, the primers were specific for *B. mandrillaris* and did not give any PCR product with HBMEC DNA (Fig. 2a, lane 5).

Next we determined the potential of this assay in the sensitive detection of *B. mandrillaris*. To achieve this, we used Chelex to extract DNA from *B. mandrillaris* and PCR amplifications were performed as described above. The DNA from as few as 100 cells could be detected with the *Balamuthia*-specific primers using the Chelex method (Fig. 2b). Overall, these assays confirmed the identity of amoebae isolated from the post-mortem specimen of the GAE patient as *B. mandrillaris*. The use of Chelex resulted in a satisfactory sensitivity for detecting the presence of *B. mandrillaris* in early cultures. For sensitive detection on clinical samples the development of a nested protocol based on the same multiple target may be necessary.

Serum from the GAE patient reacts strongly with *B. mandrillaris* (baboon isolate)

To determine the presence of *Balamuthia* antibodies in the GAE patient, antigens from *B. mandrillaris* (baboon isolate) were reacted with the patient's serum using indirect immunofluorescence assays. We demonstrated that antiserum

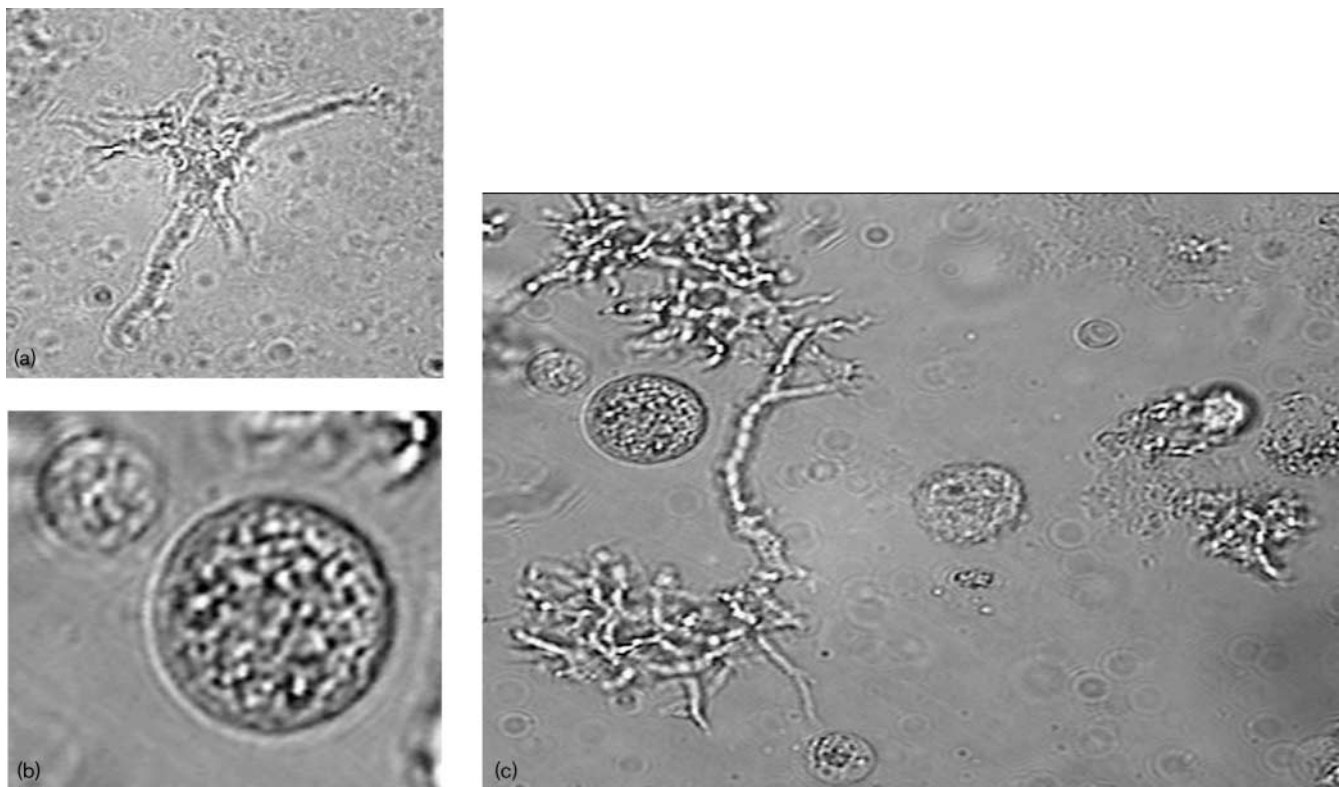


Fig. 1. Amoebae isolated using HBMECs from a granulomatous meningoencephalitis patient are *B. mandrillaris* based on morphology. Both the brain and the CSF specimens were incubated with HBMEC monolayers for several weeks as described in Methods. *B. mandrillaris* was observed by phase-contrast microscopy. (a) Trophozoite; (b) immature cyst; and (c) both. Note the large slender pseudopods (characteristic of *B. mandrillaris*, and showed visible motion). Magnification: $\times 200$ (a, c); $\times 400$ (b).

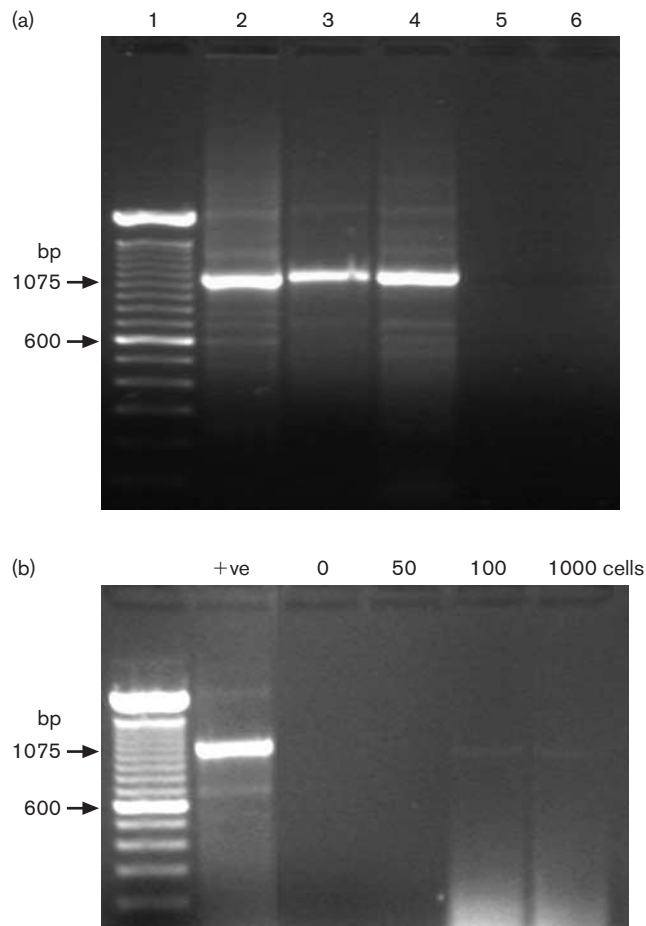


Fig. 2. (a) Identity of *B. mandrillaris* from the brain tissue and the CSF specimen of the infected patient were confirmed using PCR. Agarose gel showing amplification of mitochondrial 16S rRNA of *B. mandrillaris* as described in Methods. Lanes: 1, 100 bp DNA ladder; 2, baboon isolate of *B. mandrillaris*; 3, *B. mandrillaris* from brain tissue of the GAE patient; 4, *B. mandrillaris* from the CSF specimen of the GAE patient; 5, DNA from HBMECs; 6, negative control. Note that both the brain and the CSF amoebae gave a PCR product confirming their identity as *B. mandrillaris*. The DNA from HBMECs did not give any product, indicating the specificity of the primers. (b) Use of Chelex to extract DNA for the sensitive detection of *B. mandrillaris*. DNA was extracted from 50, 100 and 1000 cells using Chelex and used for PCR reactions as described in Methods. Note that the PCR product was observed with as few as 100 cells. *B. mandrillaris* (baboon isolate) was used as a positive control.

reacted strongly against *B. mandrillaris* with a titre of 1:10 000, further supporting the infecting organism to be *B. mandrillaris* (Fig. 3).

***B. mandrillaris* isolate from the GAE patient exhibited severe HBMEC cytotoxicity**

To determine the effects of the human isolate of *B. mandrillaris* on HBMECs and to develop an *in vitro* model for the study of *Balamuthia* pathogenesis, cytotoxicity assays were performed. Briefly, *B. mandrillaris* was incubated with

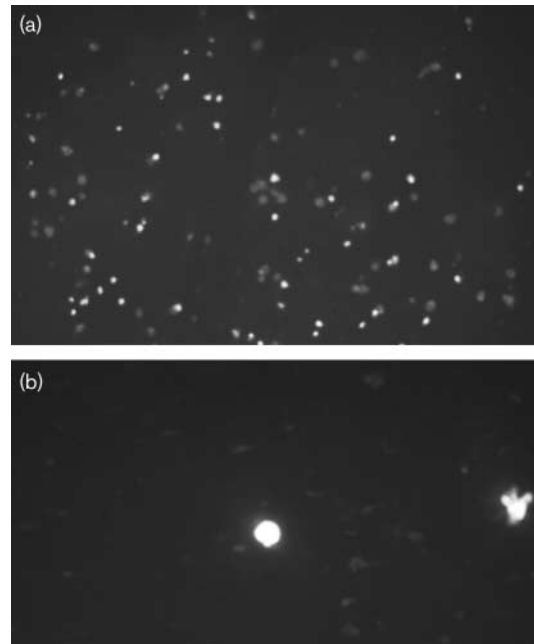


Fig. 3. Serum from the GAE patient strongly reacted with *B. mandrillaris* (baboon isolate). To determine the presence of *Balamuthia* antibodies in the GAE patient, antigens from *B. mandrillaris* (baboon isolate) were reacted with the patient's serum using indirect immunofluorescence assays as described in Methods. Note that the antiserum reacted strongly against *B. mandrillaris* from the patient with a titre of 1:10 000, further confirming the amoebae to be *B. mandrillaris*. Magnification: $\times 33$ (a) and $\times 133$ (b).

HBMEC monolayers grown in 24-well plates. We demonstrated that co-incubations of *B. mandrillaris* with the host cells resulted in severe HBMEC monolayer disruptions within 24 h ($75.7 \pm 6\%$; Fig. 4). In addition, LDH assays revealed that the human isolate of *B. mandrillaris* produced $82.5 \pm 3.2\%$ HBMEC cytotoxicity within 24 h, further confirming its pathogenic potential. This assay will be of potential value in understanding the precise mechanisms associated with *B. mandrillaris*-mediated host cell cytotoxicity.

DISCUSSION

This is the first report demonstrating the isolation of *B. mandrillaris* from a case of GAE in the UK. The patient was a young South American male, aged 33, who presented with characteristic skin lesions, acute confused state and multiple cerebral lesions involving extensive right hemispheric oedema and hydrocephalus under CT scan, which proved fatal within a few months of the onset of the disease. Previous reports have suggested that free-living amoebae which cause fatal GAE are opportunistic protozoan parasites causing infections in immunocompromised hosts. In support, almost all reported GAE infections due to *Acanthamoeba* have been reported in immunocompromised patients. The patient had no history of HIV infection or of being immunocom-

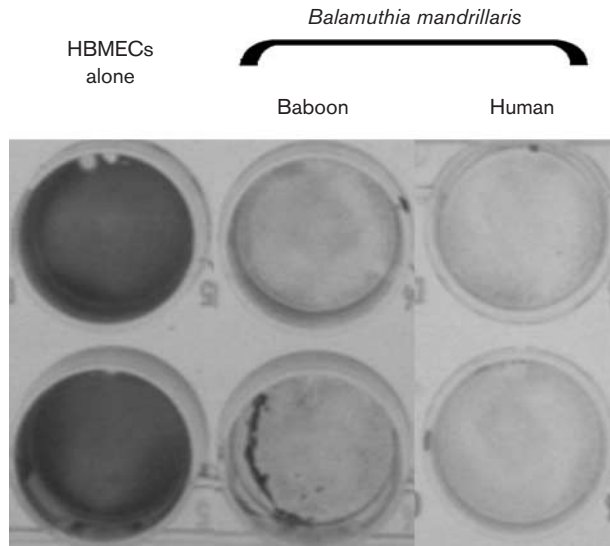


Fig. 4. Baboon and GAE isolates of *B. mandrillaris* produced severe HBMEC cytotoxicity. Both isolates (5×10^5 per well) were added to HBMEC monolayers in 24-well plates and cultures were incubated in a 5% CO_2 incubator at 37 °C for 24 h. HBMECs without parasites were used as controls. After this incubation, cytotoxicity was determined visually by haematoxylin staining. Results are representative of three independent experiments performed in duplicate. Note that both isolates produced HBMEC monolayer disruptions.

promised and his CD4 count was only slightly depressed ($621 \text{ cells } \mu\text{l}^{-1}$; normal range $775\text{--}1385 \text{ cells } \mu\text{l}^{-1}$). This finding suggests that *B. mandrillaris* can cause fatal infections in relatively immunocompetent adults and this is supported by the finding of this organism in immunologically normal children (Hansman *et al.*, 1996; Healy, 2002; Katz *et al.*, 2000; Reed *et al.*, 1997; Rowen *et al.*, 1995; Yang *et al.*, 2001), confirming the previous notion that *B. mandrillaris* is more virulent than *Acanthamoeba* (Huang *et al.*, 1999).

One of the novel findings in our study is the isolation of *B. mandrillaris* from the post-mortem CSF sample of the infected patient. To our knowledge, this is the first report describing the CSF isolation of *B. mandrillaris*. If this reflects the condition in life, it is possible that *B. mandrillaris* entry into the CNS may occur at the choroid plexus, the CSF formation site. Haematogenous spread is thought to be the route of entry, and this might take place through the highly vascular choroid plexus. The presence of multiple skin lesions on the infected patient would provide direct access to the circulation for the parasites. However, once in the circulation, parasites must interact with and penetrate the blood–brain barrier. The blood–brain barrier is highly selective, restricting the entry of pathogens, toxins, drugs as well as large molecules, and the precise mechanisms used by *B. mandrillaris* to cross the blood–brain barrier remain unclear. Further studies to understand *B. mandrillaris* penetration of the blood–brain barrier will undoubtedly help develop novel strategies to prevent and treat these serious infections.

Another interesting finding was the observation of a high *B. mandrillaris* serological titre of 1:10 000 in the infected patient. This titre is well above the range in non-infected hosts with *B. mandrillaris* titre at 1:64–1:256 (Huang *et al.*, 1999) and suggests that the patient's immune system responded strongly to the parasite's antigen(s); however, the absence of a protective response remains unexplained. This may well be due to the overwhelming infection or to the ability of *B. mandrillaris* to employ strategies to evade host immune systems. Further studies are in progress to address these issues.

Several lines of evidence suggest that early diagnosis of GAE due to *B. mandrillaris* may help successful therapy. This is supported by recent studies of Deetz *et al.* (2003), who have shown that early initiation of antimicrobial therapy resulted in successful treatment of *Balamuthia* encephalitis in two cases (a 5-year-old girl and 64-year-old man). To this end, indirect immunofluorescence has been successfully used (Huang *et al.*, 1999) and may provide a useful diagnostic tool. Similar findings were observed in this study, further supporting the use of indirect immunofluorescence as an early marker for GAE due to *B. mandrillaris*. In addition, we employed PCR using *Balamuthia*-specific primers as previously described (Booton *et al.*, 2003). A PCR product of 1075 bp was observed, further confirming the identity of the isolated amoebae as *B. mandrillaris*. However, the clinical diagnosis requires the identification of the parasites at low cell numbers. To achieve this, we made use of Chelex to isolate DNA from *B. mandrillaris*. This method does not involve washing or precipitation steps, thus eliminating loss of the DNA. Using this method, we were able to identify *B. mandrillaris* with as few as 100 cells using *Balamuthia*-specific primers. This method provides further tools for the diagnosis of *B. mandrillaris* in clinical applications.

In this study, we were able to isolate *B. mandrillaris* from both post-mortem samples including the brain necropsy and the CSF using primary HBMECs as feeder layers. Previous studies have successfully made use of African green monkey kidney cells to isolate *B. mandrillaris* from brain tissues (Schuster, 2002). However, primary HBMECs are physiologically most relevant in the actual infection as these cells constitute the blood–brain barrier. They may thus provide a useful tool for *B. mandrillaris* isolation from the CSF as well as to understand the precise mechanisms of *B. mandrillaris* passage across the blood–brain barrier, which may help develop novel strategies for therapeutic interventions.

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